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A NEW CHROMOGENIC MICROCOCCUS.

MARY HEFFERAN.

(WITH FOUR FIGURES)

In the course of an examination of river water for the Sanitary District of Chicago, carried on during the past year under the direction of Professor Jordan at the University of Chicago, a somewhat unusual opportunity has been afforded for the study of water bacteria. The water for this examination is taken from twenty-seven different stations along the Illinois and Desplaines rivers and their tributaries, and also from the Mississippi river above and below St. Louis. It is collected in sterile bottles with tightly fitting glass stoppers. As soon as the water is collected these bottles are enclosed in small tin cans which are packed with ice in larger cans, and shipped as speedily as possible to Chicago.

In making a qualitative examination of this water it has been plated in ordinary beef peptone agar (Witte's) and in Nährstoff Heyden agar.[1] The latter medium has the advantage of bringing to development a much greater number of colonies than ordinary peptone agar; for instance, at the end of ten days a peptone agar plate and the corresponding one of Nährstoff Heyden may present colonies in a ratio of 21 : 400. The reason for this enormous difference has not as yet been sufficiently determined. It is possible that the large, spreading, and rapidly growing colonies may inhibit the development of the more tardy forms on a peptone agar plate. On the Nährstoff Heyden plate these large colonies, particularly the fluorescent forms, are not seen, or at least appear only as small dots; while on the other hand, the plate is crowded with small, distinct, slowly developing colonies, some of which seem to present novel characteristics. It is likely, therefore, that this medium will furnish varieties not obtained on peptone media.

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[1] Hesse and Niedner, Zeitschr. f. Hygiene 19 : 460.

Among the germs found in this way, I isolated several times during the course of the winter a micrococcus producing a salmon-pink pigment, which seemed not to have been previously described. This form first appeared in Mississippi river water, collected at "The Chain of Rocks," near the St. Louis water tower. The water in a dilution of 1 : 100^{cc} was plated in 15 acid gelatine and peptone agar, and in neutral Nährstoff Heyden agar, using 1^{cc} of the diluted water to each plate. The micrococcus developed only on the Nährstoff Heyden plate, as it did in other experiments with the same water, although on one occasion I found it upon an ordinary agar plate, showing that the special medium is not essential. It also develops readily upon agar in pure culture. Water from other stations along the rivers often gave rise to pink colonies on peptone agar or Nährstoff Heyden, which by casual observation could not be distinguished from those of the micrococcus. A closer examination, however, usually revealed in these cases a bacillus whose pigment upon cultivation showed less of a yellow tinge in the rose than that of the salmon-pink form. On one or two occasions these small pink colonies were found to be due to a yeast, but the bacillus was by far the more common form. The micrococcus may exist in other parts of the rivers, although I did not observe it in any other water than that of the Mississippi. My search for it was only incidental in connection with other work, and the notes upon its appearance refer it always to the locality mentioned.

In the two cases in which the germ was isolated and cultivated through all the stages, it occurred in close connection with a bacillus producing a yellow pigment. The first transfer from a rather crowded Nährstoff Heyden plate or from the more isolated colony upon the peptone agar plate resulted in a mixed culture of the two kinds, a second plating being necessary in order to separate them. The bacillus was not identified. The micrococcus, which it is the purpose of this paper to describe, grows well and is easily cultivated at room temperature in broth and milk and on all ordinary solid media except potato. The cultural features are as follows :[2]

Gelatine.—On a gelatine plate at 15°–20° C. it grows slowly. The colonies were visible on the third day and appeared under low power as small irregular dots, transparent, but with a slight, thin, uniform granulation. On the next day those that had emerged upon the surface had lost their irregularity and had become perfectly circular and slightly larger; otherwise to the eye they were unchanged, no pigment being visible. Under a higher power the granulation appeared more distinct, the colonies seemed thicker and had a yellowish tinge in the center, and were thinner and more transparent at the edge. A few days later the surface colonies had taken on the characteristic salmon tinge. Under the low power they looked perfectly homogenous except for thinness at the periphery where the fine granulation showed more distinctly. The edges were smooth and clearly defined. Later only a very slow increase in size took place, the colonies reaching a few millimeters in diameter in several weeks.

Gelatine streak culture at 15°–20° C. showed growth on the third day in two delicate, finely wrinkled lines, one each side of the needle track. This was at first a pale creamy-pink in color. On the sixth day the lines were somewhat broader and had lost their delicate wrinkled appearance, the lower parts being confluent and smooth. Later the growth took on the true deep salmon color. There seemed to be almost no softening of the gelatine, except possibly a slight sinking of the line of growth.

Gelatine stab culture at room temperature showed a small cream colored colony on the surface and a slight development along the needle track at the end of the third day. The growth is very slow, the drawing (*fig. 1*) being taken from a three weeks old culture. The colony on the surface increases gradually in diameter and becomes more deeply colored, sometimes showing a slight zoning, the deeper pink in the center. The only evidence of liquefaction at first is that the surface of the gelatine sinks perhaps more rapidly than could be accounted for by ordinary evaporation, forming a hollow cup-shaped cavity lined by the smooth dry pink layer of the expanded surface colony. If the gelatine is rather stiff this surface sinking takes place very slowly. This dry cavity may reach two or three centimeters in depth in the course of a month (*fig. 2*). The deep growth along the needle track remains colorless and little developed.

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[2] Except where otherwise indicated the media used were prepared in accordance with the recommendations of the Bacteriological Committee. Jour. Amer. Pub. Health Assoc., January 1898.

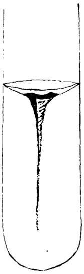
FIG. 1.

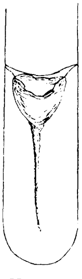
FIG. 2.

Agar.—To the eye the agar plate colonies do not differ materially from those on gelatine. They appear on the fourth day at room temperature as minute dots, which take on a yellowish tinge. Examined under low power they are seen to be very irregular, and unlike the gelatine colonies they do not become smooth edged and round upon reaching the surface, but retain their irregularity, the edges showing a slight raggedness. The texture is uniform and very finely granulated, being somewhat more dense in the center and thinner on the periphery. They become salmon-pink in the course of a few days and increase very slowly in size (*fig. 3*).

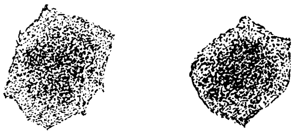
FIG. 3.

Agar streak shows beginning of development in twenty-four hours as a slight whitish growth along the needle track. In forty-eight hours it has become a light pink, a much more creamy or yellowish-pink than the rose culture of *M. agilis* of a day or two older but of about the same development. Later the growth becomes quite luxuriant, gradually deepening in tint. It has much the same appearance as the agar growth of a pink yeast, Torula III, except perhaps for a more decided salmon color. On fresh, moist agar the edges are smooth and the whole growth a straight, shining, moist layer. If the agar is older the edges are likely to be irregular or lobulated.

The reaction of the agar, ranging 15 acid, neutral, or 15 alkaline, Fuller's scale, produces little perceptible effect, although the early development may be slightly more rapid on neutral agar.

At 37° the growth is very limited, and there is no pigment, but growth proceeds normally on removal from the incubator. Light seemed to have little influence upon pigment production; cultures standing on a shelf near a north window where no sunlight entered had about the same appearance as those kept continually in a dark locker. Development on glycerine and 2 per cent. glucose agar showed no variations from ordinary growth.

Potato.—The micrococcus refused to develop on either acid or alkaline potato at room temperature or in the incubator.

Milk.—Cultures at 20° C. showed no change beyond a deposit and a thick surface ring of the same characteristic color as the agar and gelatine cultures. Lactose litmus milk brought out this color very well, the litmus itself turning a more vivid blue, indicating an alkaline reaction. In course of a month or more, the liquid showed traces of peptonization without coagulation.

Bouillon.—Became cloudy in forty-eight hours and remained so with no change beyond the slow accumulation of a salmon-pink deposit. No production of indol nor reduction of nitrate.

Fermentation tube.—Dextrose 2 per cent. showed slow growth in the bulb and accumulation of pink sediment. No cloudiness in the closed arm and no gas.

Staining reactions, morphology, etc.—The micrococcus stains readily with ordinary stains and is decolorized by Gram's method. Mounted preparations reveal small cocci measuring about 0.6 μ in diameter, grouped irregularly with no particular indication of sarcina, diplo- or strepto-coccus form. The unstained specimens in hanging drop showed no motility other than the Brownian vibration. They measure less than 1 μ, somewhat smaller than unstained specimens of *M. agilis.* No spores observed.

Upon cultivation in gelatine hanging drop under $\frac{1}{12}$ immersion lens, the cell multiplication appeared as shown in *fig. 4*. I was unable with this power to see the actual process of division, but there was little doubt that it took place in only two planes giving rise to a typical staphylococcus grouping. That division did not occur in three directions was evident from cultures in liquid gelatine or broth, in which the cells divided regularly into groups of two cells each, which remained together a short time, then separated into free single cocci. The best series observed was that figured in the drawing, the development occurring in a solid gelatine drop as follows: twenty-four hours after sowing, in which time multiplication is well begun, a group of three cells was brought into focus, carefully examined, and drawn at 11:30 A. M. I examined the group at intervals of a few minutes without seeing any signs of division, but at 2:30 it was evident that two cells had taken the place of one in the group. At 4:00 P. M. there were five cells, and two hours later (6:00 P. M.) an oblong group of six cells could be seen in one place. At 8:00 the next morning ten cells were clearly visible, and the group had begun to acquire the circular form characteristic of all the more advanced colonies in gelatine culture, although in agar hanging drop, as on an agar plate, the colony form remains irregular. At 2:30 P. M. of the second day of observation, only a slight increase had taken place, fourteen cells being in focus in one plane, forming a rosette-like figure. Afterward the growth was very slow, the last drawing of the colony having been made five days after sowing. All colonies showed the same glomerulus structure.

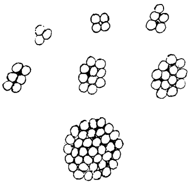

FIG. 4.

In the endeavor to identify this micrococcus with the pink varieties already described, I found some unexpected difficulties. First, the liquefaction of gelatine by this form is so slight as to pass almost without remark, making it doubtful in which of Migula's two classes the germ should be placed.[3] It is necessary, therefore, to examine his whole series, especially with regard to the two points most evidently characteristic of this form, its pigment coloration, and non-development on potato.

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[3] MIGULA: System der Bakterien **2**: —. 1900.

Migula describes eleven non-liquefying micrococci as *Rot wachsend.* Of these, seven may be thrown out, since they are distinctly said to have a seal, or carmine red, or vermilion pigment. Another is "gray on agar, red in milk, and does not grow at 37°." The other three are said to be "flesh red," the exact color of which is doubtful. Of these:

(1) *M. carneus* Zimmerman is described by Zimmerman himself[4] as having a deep flesh red, almost violet tinge, and growth on potato. Lustig describes a form, *Coccus ruber*,[5] found in water by Maschek which is probably identical with this.

(2) *M. sarcinoides* Migula (n. sp.)[6] looks often like sarcina, It is also described as a very large coccus, 1.8–2.5 μ in diameter. Nothing is said of the potato growth. Gelatine plate colonies reach 5^{mm} in diameter. Color on agar is a warm flesh red, more intense along the middle line. Bouillon has a flocculent whitish growth and a red sediment. This description, I think, hardly coincides with that of the salmon-pink form, although much depends upon what is meant by "flesh red."

(3) *M. rubellus* (Migula)[5] is morphologically scarcely distinguishable from the last, and shows the same sarcina grouping. The colonies are almost opaque, dark gray-brown. The description of the gelatine stick culture has some resemblance to that of the salmon-pink form, *i. e.*, a dry surface colony slowly spreading to walls of tube and having underneath a deep dry cavity of non-liquefied gelatine. But this growth is said to be a "bright flesh red." All cultures have a trimethylamine odor; on agar streak the edges are finely dentate, and bouillion has a red slimy sediment, enough points of difference to distinguish the forms.

In the class of germs which Migula describes as liquefying gelatine, we find out of the twelve named that three have carmine red pigment (*M. rubens*, *M. persicus* Kern, *M. haematodes* Babes); one is wine red (*M. rubiginosus* Kern); one has a violet tinge (*M. roseo-persicus*); and one (*M. fragilis* Dyar) is "brown-red on glycerine agar." These colors are not likely to be mistaken, and cut the list down to those which are either "rose" or "flesh red." Three of these (1) *M. roseus* Flügge, (2) *M. roseus* Eisenberg, and (3) *M. roseus* Maggiora are sometimes confused because of the similarity of name.

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[4] Die Bakterien unserer Trink- und Nutzwässser. Chemnitz, 1890.

[5] Diagnostik der Bakterien des Wassers 40. 1893.

[6] Op. cit.

(1) *M. roseus* Flügge is only briefly mentioned by Flügge himself,[7] but is described more at length by Macé[8] as a form common in air, non-liquefying on a gelatine plate, slowly liquefying in a stab culture. But he says that the liquid portion is tinted red "more vermilion than by *prodigiosus*." Macé also mentions among species closely related to this, *M. agilis* Ali-Cohen, *M. rouge cerise* of List, isolated from water and producing a cerise color on potato, and Zimmerman's *M. carneus* which Macé, as well as Lustig, regards as identical with Maschek's *Coccus ruber*. He describes these as all non-motile cocci, about 0.8μ in diameter, "rose red or flesh colored," and abundant on potato.

(2) *M. roseus* Eisenberg,[9] to which Migula gives the name of *M. subroseus*, is found in sputum and produces a pigment like that of *B. prodigiosus* on potato.

(3) *M. roseus* Maggiora is described by Ward[10] as a non-liquefying form 0.6μ in diameter, associated in irregular glomeruli, and forming a pale rose pigment. I was unable to gain access to a further description of this micrococcus.

As to the remainder of Migula's list there is left:

(4) *M. cumulatus* Kern, a too definitely liquefying form to be identified with the salmon-pink micrococcus. Otherwise, excepting the fact that it is a facultative anaerobe, the descriptions are somewhat similar.

(5) *M. subcarneus* Migula shows in a gelatine stab culture after one day a small light red colony at point of incision. After three weeks a sirupy liquefaction begins in stocking-shaped

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[7] Die Mikroorganismen 185 [3d ed].

[8] Traité practique de bactériologie 432. 1891.

[9] Bakteriologische Diagnostik 408. 1891.

[10] Annals of Botany **12**: 309. 1898.

form, with the colony on the surface and a thick, flocculent, rose-colored sediment. On agar, "rose" or "flesh colored."

(6) *M. rosaceus* (Frankland) is found in air. The entire description is as follows:[11] Cocci which are very variable in size, the largest 2.5 μ in diameter. On gelatine forms a smooth pink expansion on the surface, while needle track below remains almost undeveloped. In older cultures the margin assumes a radiated appearance, while still older cultures frequently exhibit slight liquefaction. On agar, smooth bright pink surface expansion, devoid of any further character. Broth after nine days is clear, free from pellicle, and has a pink deposit. On plate, the colonies appear to the naked eye as pin-head dots on surface, and are bright pink in color. Under low power (× 100) are seen to be of distinctly reddish tint, the edge irregular but smooth. As the colonies approach the surface, the irregularity diminishes.

Certain points in this description agree somewhat with that of the Mississippi river form, *i. e.*, the gelatine culture and plate colonies, but the size of the coccus, the agar and broth culture seem to differ, and the description is too imperfect to make the comparison at all complete. The same may be said of Frankland's description of his (7) *M. carnicolor*, the only stated points of difference from (6) being (*a*) a more rapid growth, (*b*) fainter color of pigment, and (*c*) slightly different appearance of colonies (more circular and brownish-pink). In both these forms the bouillon cultures are said to be clear after nine days, while numerous cultures of the salmon-pink form have remained cloudy for weeks.

This concludes the list of red growing cocci which I have found described, except that mention might be made of Bumn's *Diplococcus roseus* and *Sarcina rosea* (Schroeter), both liquefying air forms;[12] also *Sarcina mobilis* (Maurea),[13] which liquefies and will not develop on potato, but has a brick-red pigment. I have already mentioned *M. agilis* in the body of the description.

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[11] Phil. Trans. Roy. Soc. London **178**: 269. 1887.

[12] EISENBERG, op. cit., 12 and 16.

[13] STERNBERG, Manual of Bacteriology 720. 1893.

It is evident that the color of the pigment produced by these germs, otherwise so similar, is of some importance in an effort to distinguish them. The descriptions are, for the most part, very meager in actual definite points, and it is doubtful if even the twenty-six standard tests given by Fuller[14] as showing a high degree of constancy would serve to differentiate these forms, so general in character are the indicated reactions of the table. In regard to the chromogenesis, it would be of no advantage to multiply the list of adjectives, for few bacteriologists could describe in this way the exact tints of "pink," "reddish" "rose," or "flesh color" with sufficient accuracy to make them unmistakable. An article by E. B. Shuttleworth,[15] on "Nomenclature of colors for bacteriologists" is one of the most recent attempts to systematize colors for bacteriology. This author's classification gives under yellowish-pink three subdivisions: salmon, salmon-buff, and flesh color. I was unable, however, to obtain the plates or illustrations to which his numbers evidently referred, and without them the words do not give a very distinct visual image.

A simple and more exact method of determining the pigment color of germs is that of the use of a color wheel. A little contrivance of the kind often used in morphology and easily obtained is a wooden top with the various small color disks. A better determination, because of the more constant rate of revolution of the disks, can be made with a Bradley color wheel and standard Maxwell disks. By this means I obtained the following result in the case of the salmon-pink germ.

An agar culture begins in a cream-pink growth for which the determination is not necessary. After about 5–7 days it begins to acquire the characteristic color, the composition of the color wheel for this age being near: red, 70 per cent.; white, 20 per cent.; yellow, 10 per cent.. Later the color deepens with the addition of a more orange tone, and remains almost constant after two weeks for all culture media—agar (different reactions), gelatine, on which the orange tint is slightly more marked, broth sediment, milk ring. Several trials to match this with the color wheel resulted in a range of:

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[14] Jour. Expt. Med. 4: 609. 1899.

[15] Jour. Amer. Pub. Herath. Assoc. 1895: 406.

	(1)	(2)	(3)
Red	76%	72%	72%
White	10	14	13
Yellow	8	9	9
Orange	6	5	6
	100	100	100

Of these, (3) was almost the exact tint of my cultures, with the one difference of a slightly duller tone than that shown by the actual growth, particularly of a rather bright gelatine culture. But any one of these combinations gives a good idea as to the characteristic tint.

By this method of description of colors there would be less possibility of confusion. The great advantage would lie in the systematization of those chromogenic germs whose color is fairly constant on ordinary media for certain ages. For instance, the whole *prodigiosus* group produces a color in liquefied gelatine which may be variously described in the vocabulary of different bacteriologists; but the fact that it closely approximates a color combination on the wheel of red 80 per cent., black 20 per cent., is sufficiently definite. *B. prodigiosus* and *B. ruber balticus* both give the above color to liquefied gelatine in a ten-day culture. In addition to this *B. ruber balticus* produces a heavy surface membrane of an entirely different shade, *i. e.*, red 80 per cent., orange 20 per cent.

The one difficulty for this accurate determination of pigment color is, of course, the liability of variation in the pigment produced by the same germ on media made by different bacteriologists or in cultivation under different circumstances. However, it has been found that if this organism is brought into good physical condition by a series of broth and gelatine cultures, according to Fuller's suggestion,[16] such variations in standard media are not of very wide range.

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[16] Jour. Expt. Med. 4:609. 1889.

Because of the peculiar salmon or yellowish-pink color of the pigment produced by the organism described in this paper, and because, although it is evidently closely allied to some of the pink micrococci previously described, it is not identical with any of them, I suggest for it the name **Micrococcus roseus flavus.**

In conclusion, I wish to express my sincere thanks to Professor Edwin O. Jordan for many valuable criticisms and suggestions during the progress of this work.

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